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IRON-ASSOCIATED OUTER MEMBRANE PROTEINS OF MAGNETIC
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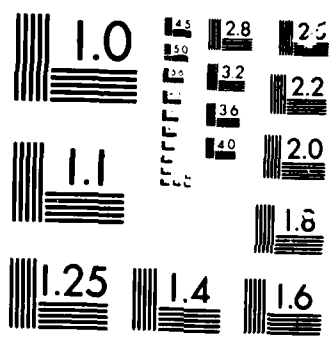
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ANNUAL LETTER REPORT

ONR Contract N00014-85-K-0502

Iron-Associated Outer Membrane Proteins of Magnetic Bacteria
University of New Hampshire

Department of Microbiology, Durham, New Hampshire 03824-3586

Richard Blakemore, P.I.

PROJECT ABSTRACT:

Iron-associated cell outer membrane proteins (FeOMPS) of magnetic bacteria are the focus of this research. In addition to determining effects of iron and oxygen supply on FeOMPs, several potential roles of these external cell proteins will be investigated to determine how they may participate in (1) iron binding and reduction to mobilize it inward (2) detoxification of activated forms of O_2 and (3) the synthesis and/or release of a soluble c-type CO-binding cytochrome. The relationship of FeOMPs to cell magnetite precipitation will be explored with the use of mutant strains. An effort will be made to document the principal cellular location and role in cell physiology and magnetosome formation of a CO-binding soluble c-type cytochrome which is selectively released from cells of a magnetic spirillum by freezing and thawing.

CONTRACT SUPPORTED PUBLICATIONS AND ABSTRACTS:

A. REFEREED PAPERS:

1. Blakemore, R.P., N.A. Blakemore, D.A. Bazyllinski and T.T. Moench. (in press) The Magnetotactic Bacteria. Bergy's Manual of Systematic Bacteriology. vol 3.

2. Paoletti, L.C., and R.P. Blakemore. (in press) Hydroxamate production by Aquaspirillum magnetotacticum J. Bacteriol.

3. Blakemore, R.P., N.A. Blakemore and R.B. Frankel. (accepted) Bacterial Biomagnetism and Geomagnetic Field Detection by Organisms. Chapter in A.A. Marino (ed.) Handbook of Bioelectricity, Marcel Dekker Inc. New York.

B. ABSTRACTS of PAPERS at MEETINGS:

1a. Paoletti, L.C., K.A. Short and R.P. Blakemore 1986. Freezing and thawing of Aquaspirillum magnetotacticum releases periplasmic proteins. Abstr. 86th Annu. Mtg. Am. Soc. for Microbiol. Wash., D.C.

2a. Paoletti, L.C. and R.P. Blakemore 1985. Hydroxamate production by a magnetic spirillum at high but not low iron. 2nd Annu. NEMPET mtg. Ithaca, N.Y. (a regional physiology mtg.).

3a. Paoletti, L.C. and R. P. Blakemore 1986 Antigenic relatedness of a periplasmic c-type cytochrome and an outer membrane in Aquaspirillum magnetotacticum. 3rd Annu. NEMPET mtg. Woods Hole, MA (a regional physiology mtg.).

4a. Gorby, Y., T. Beveridge, and R.P. Blakemore 1986. Ultrastructure and composition of the magnetosome sheath. 3rd Annu. NEMPET mtg. Woods Hole, MA

MEETINGS ATTENDED:

1. American Society for Microbiology March, 1986 Washington D.C.
2. Northeast (mtg) of Ecologists, Morphologists, Physiologists and Taxonomists (NEMPET) 3rd Annu. Mtg., Woods Hole, MA. June, 1986 (a regional microbiologists mtg)

PERSONNEL:

Name:	Approx. ONR Support
1. Richard P. Blakemore, - P.I.	2.0 mos.
2. Nancy A. Blakemore, - Tech III	9.5 mos.
3. Y.A. Gorby, - Grad. Proj. Assistant	2.0 mos.
4. L. C. Paoletti - Grad. Proj. Assistant	7.0 mos.
5. K. Short - Grad. Proj. Assistant	11.0 mos.

PROJECT PROGRESS SUMMARY:

The attached abstracts of papers (Appendix I) summarize many of our recent findings.

Freezing and thawing cells of Aquaspirillum magnetotacticum selectively released cell periplasmic proteins (ref 1a, cited above). C-type hemes were released from magnetic spirilla by the simple freeze/thaw procedure (ref 3a and previous references cited therein), with surprisingly few other proteins. Thus, these are now known definitively to be periplasmic substances. The c-type hemes include an 85 kdal and a 16.5 kdal pink protein. The former appears to be the c-moiety of a cd₁ nitrite reductase whereas the other appears to bind carbon monoxide and has peroxidase activity. Studies of the properties of each of these c-type hemes are in progress. The latter may be an important O₂-detoxifying enzyme in this (catalase-negative) species. As such, its location in the periplasm and cell outer membrane (see below) would be especially advantageous in this obligate microaerophile.

Antisera raised in rabbits immunized with the purified 16.5 kdal hemoprotein, cross-reacted in Western Blots of cell outer membrane proteins thereby indicating antigenic relatedness between the 16.5 kdal periplasmic hemoprotein and a protein of similar mol. wt. in the cell outer membrane (ref 3a). This

confirmed the presence of c-type cytochromes in the cell outer membrane. Because c-type hemes have frequently been used as markers of cell inner membranes in cell fractionation studies, our unexpected and interesting findings should be extended. One goal will be to identify or confirm role(s) of these cytochromes in their unusual cell locations.

Aquaspirillum magnetotacticum cells grown at high but not low iron produced hydroxamate material which served as an iron chelator for a siderophore-deficient Salmonella strain. (refs 2 and 2a). This provides the first evidence for iron chelators in magnetic bacteria and indicates an unusual pattern of hydroxamate release - at high but not low iron. Cells apparently require at least 5 μ m Fe to induce synthesis of siderophores and continue to produce them at concentrations of iron found in their natural habitats (20 μ m). This is very different from the pattern of siderophore production observed in enteric bacteria.

The magnetosome sheath has been shown to behave in freeze-etching studies as a lipid bilayer (ref 4a). The protein and lipid composition of the magnetosome sheath is currently under study.

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APPENDIX

(REPRINTS OF SELECTED ABSTRACTS)

Hydroxamate Production by *Aquaspirillum magnetotacticum*

LAWRENCE C. PAOLETTI AND RICHARD P. BLAKEMORE*

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Received 4 February 1986/Accepted 11 April 1986

Spent culture fluids from *Aquaspirillum magnetotacticum* MS-1 grown at high (20 μ M) but not low (5 μ M) iron concentration contained material yielding a positive hydroxamate test. Cells possessed six major outer membrane proteins. Three outer membrane proteins ranging from 72,000 to 85,000 daltons were coordinately produced at iron concentrations appropriate for hydroxamate production. A 55,000-dalton iron-repressible outer membrane protein was also present in strain MS-1 cultured at low but not high ferric quinate concentration. Culture fluids from strain MS-1 which were hydroxamate positive augmented growth of a *Salmonella typhimurium* siderophore-deficient (*enb-7b*⁻) mutant in low-iron medium, suggesting a role of hydroxamate in uptake of iron by the cell.

Numerous bacterial proteins, including cytochromes, catalases, peroxidases, superoxide dismutases, ribotide reductases, and nitrogenases, contain iron (15). Because of its insolubility at neutral pH under aerobic conditions, iron is usually unavailable for direct uptake by cells (16). Under conditions of low iron concentration (less than 1 μ M), many microorganisms produce iron chelators, termed siderophores (16). These are assimilated into gram-negative cells by means of specific receptor proteins located in the outer membrane (16, 18, 20).

Siderophores have been detected in spent culture fluids from aerobes and facultative anaerobes but are apparently not produced by strict anaerobes or the lactic acid bacteria (15, 16). No information exists concerning siderophore production by obligate microaerophiles.

Aquaspirillum magnetotacticum (13) is a gram-negative, obligately microaerophilic chemoheterotroph, 2.0% of which (dry weight) is iron. Although proteins and hemoproteins of this organism contain iron, most of this metal is compartmentalized within its magnetosomes, which are intracellular enveloped crystals of the iron oxide magnetite (2). Virtually nothing is known of the manner in which cells of this organism sequester iron. However, in both its natural habitat and its culture medium the total iron concentration is 20 μ M. In nature, the iron may be complexed with humic substances or plant-derived organic acids. In the culture medium used, iron is chelated with quinic acid.

This study was initiated to determine whether *A. magnetotacticum* uses a high-affinity (siderophore) system similar to those used by other gram-negative organisms for iron acquisition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Cells of *A. magnetotacticum* MS-1 (ATCC 31632) and of a nonmagnetic mutant *A. magnetotacticum* strain, NM-1A, were cultured microaerobically in chemically defined growth medium (MSGM) as previously described (3). The iron source, ferric quinate, was provided at concentrations of 0, 5, 10, 20, or 40 μ M. FeSO₄ was omitted from the culture medium mineral solution, and for studies involving spectrophotometric analysis of supernatant fluids, resazurin was omitted. A ferric chloride-sodium citrate mixture with a citrate-to-iron molar

ratio of 1:1 or 20:1 (an iron concentration of 20 μ M) was used in lieu of ferric quinate. Without added iron, MSGM contained 0.35 μ M iron, as determined by the ferrozinc method (19). No attempts were made to completely remove iron from the culture medium.

Salmonella typhimurium LT-2 *enb-7b*⁻, an enterobactin-deficient mutant (a gift from J. B. Neilands, University of California at Berkeley), and *S. typhimurium* ATCC 14028 were maintained on nutrient agar slants and subcultured bimonthly. To promote siderophore production, *S. typhimurium* ATCC 14028 was cultured for 48 h at 37°C on a rotary shaker in 0.25% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.) solution containing 0.2 mM MgCl₂ and adjusted to pH 7.5 (18).

Isolation of outer membrane proteins. *A. magnetotacticum* MS-1 and NM-1A were grown to early stationary phase in 1-liter batch cultures. Cells were harvested by centrifugation (7,000 \times g for 15 min at 4°C) and suspended in 10 ml of 50 mM potassium phosphate buffer (pH 6.8). Outer membrane proteins (OMPs) were isolated by the procedure of Schnaitman (17). Briefly, DNase and RNase (Sigma Chemical Co., St. Louis, Mo.) were each added to cell suspensions at a final concentration of 0.1 mg/ml. Cells were ruptured by two passes through a precooled French pressure cell (16,000 lb/in²). Unbroken cells and cellular debris were removed by centrifugation (7,000 \times g for 15 min at 4°C). The resulting supernatant fluid was ultracentrifuged at 200,000 \times g for 60 min at 4°C. The brown pellet, containing both inner and outer cell membranes, was suspended in 10 ml of 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.4) containing 2% (vol/vol) Triton X-100 and 10 mM MgCl₂. The unsolubilized outer membrane fraction was collected by ultracentrifugation (200,000 \times g for 60 min at 4°C) and washed once in 10 mM HEPES (pH 7.4) to removed residual Triton X-100. The solubilized cytoplasmic membrane proteins were precipitated with cold 95% ethanol overnight at -72°C and collected by centrifugation (7,000 \times g for 30 min at 4°C). Protein determinations were made by the procedure of Lowry et al. (11) with bovine serum albumin as a standard. The activity of succinic dehydrogenase, a cytoplasmic membrane enzyme, was assayed in each cell fraction (5) to assess the purity of the outer membrane fraction.

Electrophoresis and analysis of OMPs. OMPs and molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were solubilized and separated by the electrophoretic

* Corresponding author.

B.2a

Freezing and Thawing of *Aquaspirillum magnetotacticum* Release
Periplasmic Proteins

L. C. PAOLETTI, K. A. SHORT AND R. BLAKEMORE

16 86TH Annual ASM Meeting - WASHINGTON, D. C.

ABSTRACT

FREEZING AND THAWING (F/T) CELLS OF AQUASPIRILLUM
MAGNETOTACTICUM STRAIN MS-1 IN 10-50 MM POTASSIUM PHOSPHATE (PH
6.8) OR HEPES (PH 7.4) BUFFER RESULTED IN SELECTIVE RELEASE OF
PROTEINS. AFTER THAWING, CELLS WERE NOT VISIBLY DISRUPTED AND
SOME WERE MOTILE. THE F/T SUPERNATANT FLUID CONTAINED 60
PROTEINS AS DETECTED BY SILVER STAINED SDS-PAGE. NON-SDS GELS
EXHIBITED 6 PROTEINS WITH SUPEROXIDE DISMUTASE AND 4 WITH
PEROXIDASE ACTIVITY. THE PINK F/T WASH FLUIDS ALSO CONTAINED C-
TYPE CYTOCHROMES INCLUDING A DIHEME C_{D1}-TYPE AS DETERMINED FROM
DIFFERENCE SPECTRA. THE PAGE PROTEIN PROFILE OF F/T WASH FLUIDS
WAS NEARLY IDENTICAL TO THAT OF PERIPLASMIC FRACTIONS OBTAINED BY
CONVENTIONAL METHODS INCLUDING CHLOROFORM TREATMENT OR OSMOTIC
SHOCK. IT WAS DISTINCT FROM THOSE OF EITHER OUTER OR CYTOPLASMIC
MEMBRANE FRACTIONS. F/T FLUIDS HAD THE LOWEST SUCCINIC
DEHYDROGENASE ACTIVITY (2.9 % OF THE RELATIVE ACTIVITY DETECTED)
OF ANY ISOLATED PERIPLASMIC FRACTION. THUS, IT APPEARS THAT F/T
SELECTIVELY RELEASED PERIPLASM FROM THIS SPIRILLUM WITHOUT
DISRUPTING THE CYTOPLASMIC MEMBRANE.

RESULTS AND DISCUSSION

THE CYTOPLASMIC MEMBRANE FRACTION OBTAINED WITH THE TRITON X-100/MGCL₂ METHOD (TABLE 1), CONTAINED THE HIGHEST SDH SPECIFIC ACTIVITY DETECTED, WHEREAS THE OUTER MEMBRANE AND PERIPLASMIC + CYTOPLASMIC FRACTIONS EACH CONTAINED 8 % OF THE MAXIMUM SDH SPECIFIC ACTIVITY DETECTED.

FREEZING AND THAWING RELEASED PROTEINS FROM CELLS OF A. MAGNETOTACTICUM WITHOUT APPARENT CELL DAMAGE. THE CELL FRACTION OBTAINED USING F/T CONTAINED 2.9 % OF MAXIMUM SDH SPECIFIC ACTIVITY DETECTED. THE CELL FRACTIONS OBTAINED USING CHLOROFORM OR LYSOZYME-EDTA, CONTAINED 4.2 AND 3.1 %, RESPECTIVELY. THESE RESULTS SUGGEST THAT PERIPLASM OBTAINED BY CONVENTIONAL METHODS OR BY F/T IS RELATIVELY UNCONTAMINATED WITH CYTOPLASMIC MEMBRANE PROTEINS.

A MAJORITY (85 %) OF THE TOTAL CYTOCHROME CONTENT OF DENITRIFYING A. MAGNETOTACTICUM CONSISTS OF C-TYPE HEME. SOLUBLE C-TYPE HEMES COMPRISE 60 % OF THE TOTAL CYTOCHROMES AND INCLUDE A CD₁ MULTHEME (NITRITE REDUCTASE). PERIPLASMIC FRACTIONS, REGARDLESS OF THE METHOD USED TO OBTAIN THEM, DISPLAYED SPECTRAL CHARACTERISTICS OF C-TYPE HEMES. F/T AND PERIPLASM + CYTOPLASM (TRITON X-100/MGCL₂ METHOD) FRACTIONS, WHEN CONCENTRATED, WERE PINK-ORANGE IN COLOR AND EXHIBITED RED-OX ABSORBANCE MAXIMA (551, 522 AND 425 NM) CONSISTENT WITH THOSE OF C-TYPE HEMES (FIG. 1). THERE ARE NUMEROUS REPORTS OF SOLUBLE BACTERIAL C-TYPE

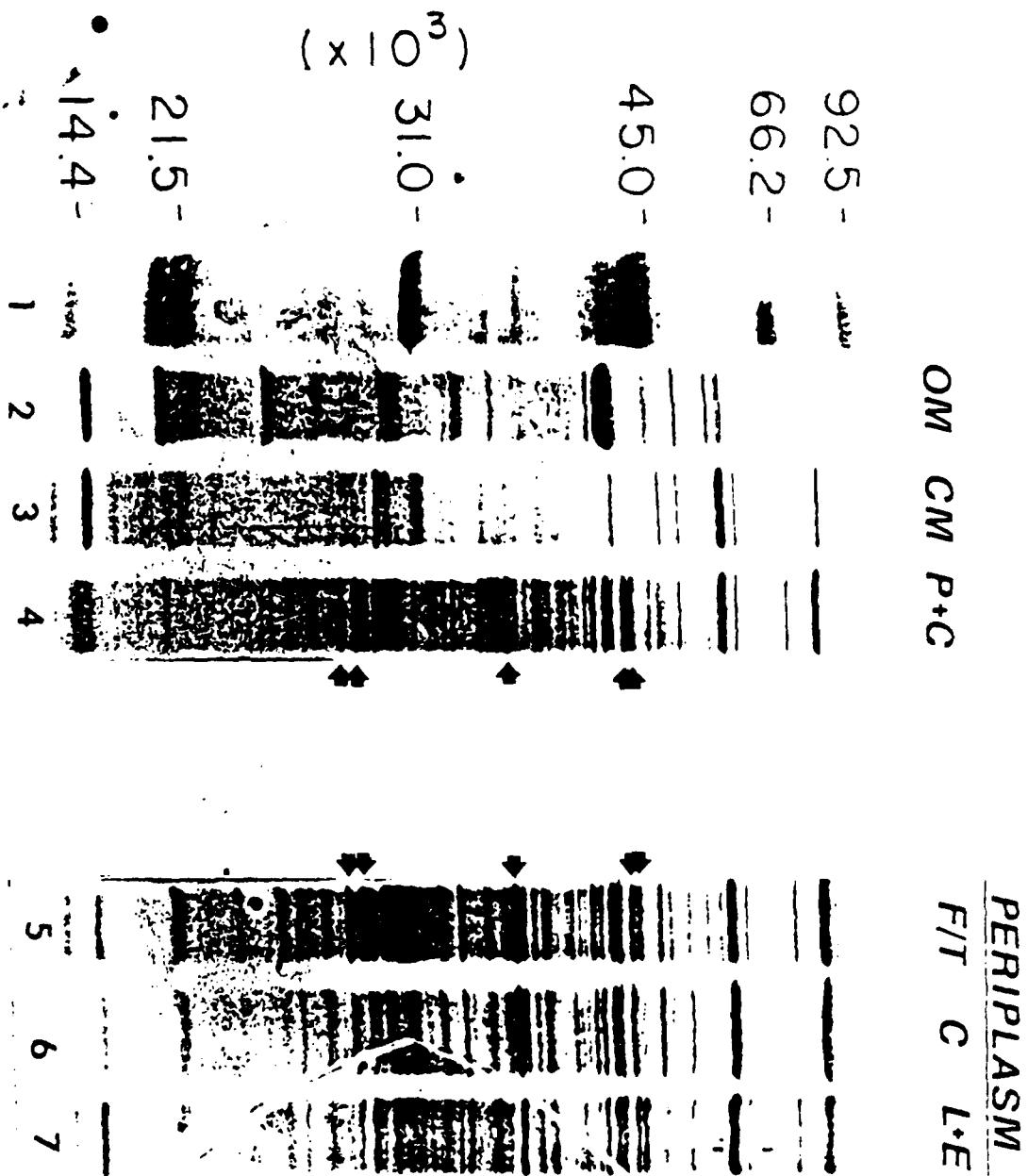
B.2a.

CYTOCHROMES (7,8,16,18). THESE FREQUENTLY APPEAR TO ORIGINATE FROM THE PERIPLASM OR TO BE LOOSELY ASSOCIATED WITH THE CYTOPLASMIC MEMBRANE. THESE HEMOPROTEINS IN A. MAGNETOTACTICUM MAY BE LOOSELY ASSOCIATED WITH THE CYTOPLASMIC MEMBRANE AND LIBERATED INTO THE PERIPLASM BY F/T.

PROTEINS OF SIMILAR MOLECULAR WEIGHT RELEASED FROM A. MAGNETOTACTICUM BY EITHER LYSOZYME-EDTA OR CHLOROFORM TREATMENT SHOWED A HIGH DEGREE OF SIMILARITY TO THOSE RELEASED BY THE F/T PROCEDURE AS DEMONSTRATED BY SDS-PAGE. EACH PERIPLASMIC FRACTION (FIG. 2, LANES 4-7) EXHIBITED OVER 60 BANDS. MANY OF THESE PROTEINS RANGING BETWEEN 29,000-42,000 DALTONS WERE PRESENT ONLY IN PERIPLASM FRACTIONS (FIG. 2, LANES 4-7). THESE MIGHT INCLUDE PROTEINS WITH KNOWN FUNCTIONS IN OTHER SPECIES (E.G. ENZYMES, BINDING PROTEINS). OUR RESULTS SHOW THAT THESE INCLUDE SEVERAL PROTEIN BANDS WITH SOD ACTIVITY AND 6 WITH PEROXIDASE ACTIVITY (FIG. 3).

THE F/T METHOD WE USED WITH A. MAGNETOTACTICUM HAS MANY ADVANTAGES OVER CONVENTIONAL TECHNIQUES USED TO RELEASE PERIPLASM. THESE INCLUDE; THE ABSENCE OF ANY CHEMICAL TREATMENT BY LYSOZYME, CHLOROFORM, TOLUENE OR EDTA; THE RAPID RECOVERY OF ENZYMES; LACK OF APPARENT DAMAGE TO THE CELL.

B.2a



B.3a.

For NEMRET - Woods Hole
June, '86

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Antigenic Relatedness of a Periplasmic c-type Cytochrome and an
Outer Membrane Protein in Aquaspirillum magnetotacticum.

Aquaspirillum magnetotacticum strain MS-1 possesses a 16,500 dalton (16.5 kdal) protein in each cellular fraction (soluble, outer membrane and cytoplasmic membrane) as visualized by silver-stained SDS-PAGE. This protein recovered from soluble cell fractions, this protein displayed spectral characteristics of a c-type cytochrome. A protein of similar size in cytoplasmic fractions was regarded as "membrane-bound" cytochrome c. The presence of an outer membrane protein (OMP) similar in mass to both the soluble and membrane-bound cytoplasmic hemoproteins was intriguing because bacterial OMPs are not known to include cytochromes. To determine if the 16.5 kdal OMP was similar to the soluble cytochrome c, cellular fractions of strain MS-1 were subjected to SDS-PAGE, transferred to nitrocellulose and probed (e.g. Western blot) using antisera raised against the soluble heme.

The 16.5 kdal OMP from cells of strain MS-1 cross reacted with antibodies raised against the purified, soluble cytochrome c. ^{See Panel C} This OMP however, did not react with antibodies raised against a soluble 82 kdal (pink) protein, believed to be the heme c of the cd_1 cytochrome complex. ^{Panel B} An 85 kdal protein (putative cytochrome cd_1 or nitrite reductase), detected in soluble and cytoplasmic cell fractions did react with antibodies raised against the soluble 16.5 kdal cytochrome c, however. ^{Panel C} Numerous soluble proteins cross reacted with antibodies raised against the 16.5 kdal cytochrome, suggesting a highly conserved peptide sequence in these and the soluble hemoprotein. Since A. magnetotacticum is an obligate microaerophile, it is conceivable that the 16.5 kdal OMP may function as a peroxidase with a principle role in oxygen detoxification, as has been demonstrated with some c-type cytochromes in other organisms. Thus, this study revealed an outer membrane component antigenically similar to a soluble cytochrome c in the magnetic spirillum. These results may lead to ^{an appreciation of} new and interesting functions of the bacterial outer membrane.

B.4a.

For NEMPET - Woods Hole
June, '86

Y A Gorby and R. P. Blakemore :

Ultrastructure and Composition of the Magnetosome Envelope

Magnetic bacteria synthesize intracellular magnetite (Fe_3O_4) particles which impart a magnetic character to cells containing them. Aquaspirillum magnetotacticum cells each contain enveloped magnetite particles termed "magnetosomes". By means of freeze etching and transmission electron microscopy, we have obtained evidence consistent with the possibility that the magnetosome sheath in this organism consists of a lipid bilayer. Results of polyacrylamide gel electrophoresis applied to magnetosomes purified by magnetic separation from disrupted cells confirmed the presence of associated proteins. These findings suggest that the magnetite within this organism is normally enveloped by a biological membrane consisting of a lipid bilayer admixed with protein.

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